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TP53 alterations and patterns of carcinogen exposure in a U.S. population-based study of bladder cancer

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The molecular pathology of bladder cancer has been the subject of considerable interest, and current efforts are targeted toward elucidating the interrelationships between individual somatic gene loss and both etiologic and prognostic factors. Mutation of the *TP53* gene has been associated with more invasive bladder cancer, and evidence suggests that *TP53* mutation, independent of stage, may be predictive of outcome in this disease. However, there is no consensus in the literature that bladder carcinogen exposure is associated with inactivation of the *TP53* gene. Work to date has been primarily hospital based and, as such, subject to possible bias associated with selection of more advanced cases for study. We examined exposure relationships with both *TP53* gene mutation and TP53 protein alterations in a population-based study of 330 bladder cancer cases in New Hampshire. Tobacco smoking was not associated with TP53 alterations. We found a higher prevalence of *TP53* inactivation (*i.e.,* mutation and nuclear accumulation) among hair dye users (odd ratio [OR] = 4.1; 95% confidence interval [CI] 1.2–14.7), and the majority of these mutations were transversions. Men who had "at risk" occupations were more likely to have mutated *TP53* tumors (OR = 2.9; 95% CI 1.1–7.6). There also was a relative absence of *TP53* mutation (OR = 0.4; 95% CI 0.0–2.9) and TP53 protein alterations (OR = 0.6; 95% CI 0.3–1.4) in bladder cancers from individuals with higher arsenic exposure. Our data suggest that there is exposure-specific heterogeneity in inactivation of the TP53 pathway in bladder cancers and that integration of the spectrum of pathway alterations in population-based approaches (capturing the full range of exposures to bladder carcinogens) may provide important insights into bladder tumorigenesis.
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Approximately 60,000 new cases of bladder cancer will be diagnosed in the United States in 2004.[1] This disease occurs predominantly in Caucasians, with other racial/ethnic groups having at least a 50% lower incidence rate. Regardless of racial or ethnic group, the male to female ratio of bladder cancer incidence generally ranges between 3 to 1.[1] Bladder cancer occurs overwhelmingly in one histologic cell type (transitional cell), although squamous cell carcinoma and adenocarcinoma of the bladder occur rarely.[2] The identified causes of bladder cancer include tobacco use, occupational exposures (in particular to aromatic amines), ingestion of arsenic and cyclophosphamide.[2–5] Disinfection byproducts, urinary tract infection and hair dyes have also been suggested as possible causes of bladder cancer, whereas high fruit and vegetable consumption, fluid intake and certain analgesics and barbiturates have been reported as protective factors.[2] Interestingly, there is marked urban-rural difference in the incidence rates of bladder cancer, and in the U.S., there is a striking persistence of elevated mortality rates in New England that remains etiologically obscure.[6]

The most frequently documented somatic genetic alteration in bladder cancer is in the *TP53* gene, with the prevalence of alterations reported varying from 14%[10] to 61%[7] with the population-based series reporting lower prevalence rates. Most studies of *TP53* alteration have been derived from relatively small, hospital-based series,[8–12] capturing higher stage patients undergoing surgery for their disease. New efforts have begun to establish population-based studies with biologic materials,[13] and data from these studies clearly indicate an association between the presence of *TP53* alterations with more advanced stage and grade of disease. As yet, relatively few studies of bladder cancer have compared specific carcinogen exposures with the character of *TP53* alteration,[8,14–17] despite the success of this approach for other types of solid tumors (*e.g.,* hepatocellular carcinoma, skin and lung cancers). Specifically, the precise nature of *TP53* alteration has helped elucidate the role of specific carcinogen exposures among subsets of cases.[18] Such studies strengthen the evidence for causality and may aid translation of mechanistic data into prevention strategies. Therefore, as part of a population-based case-control study of bladder cancer in New Hampshire, we sought to investigate the patterns of *TP53* inactivation associated with potential carcinogenic exposures,[19] studying 330 bladder cancer cases.

Material and methods

Bladder cancer cases

Residents of New Hampshire ages 25–74 years, diagnosed from July 1, 1994 to June 30, 1998, were identified by the rapid reporting system of the New Hampshire State Cancer Registry.[20] Briefly, by state law, practitioners are required to provide a report of incident cancer upon its diagnosis. Study participants underwent a personal interview to obtain information on demographic traits, use of tobacco (including frequency, duration and intensity of cigarette smoking) and use of hair dyes. Prior to the interview, participants completed a personal residence and work-history calendar. Each job was then coded according to the Standard Occupational Classification (SOC) system (U.S. Department of Commerce, 1980). Pathology material from a total of 330 patients was completely analyzed (see below). We collected and analyzed a toenail clipping sample for trace elements including arsenic using instrumental neutron activation analysis.[21] The normal growth rate for toenails is 3–12 months, and there is evidence that toenail measures of arsenic are reproducible over a period of several years.[22,23]

Pathology reports and paraffin-embedded tumor specimens were requested from the treating physician/pathology laboratories.

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Bladder tumors were reviewed by the study pathologist and classified according to the WHO classification of bladder tumors. DNA was extracted as previously reported.[24] Briefly, three 20 μm sections were cut and transferred into tubes with digestion buffer. After microwave treatment and centrifugation, the paraffin ring was removed. Paraffin-free tissue pellets were suspended in proteinase K digestion buffer. Supernatants containing DNA lysate were boiled to denature any residual protease (100°C for 10 min) and stored at −20°C. All pathology materials were labeled with a research identification number that did not reveal any personal characteristics or exposure information about the study subjects.

Immunohistochemistry

Immunohistochemical staining of paraffin-embedded slides was performed using the avidin-biotin complex technique. For each case, a single representative slide was selected for staining and histologic evaluation. Briefly, slides were deparaffinized and hydrated into water. Slides underwent antigen retrieval in Citra solution using the Biocare Decloaking Chamber (Biocare Medical, Walnut Creek, CA). Staining of p53 was performed using a monoclonal antibody (BioGenex, San Ramon, CA) at a 1:100 dilution on the Optimax I-6000 Immunostainer (BioGenex). An appropriate positive control was used in each staining run, and each slide was stained with a negative control. The percentage of positively staining tumor cells was scored (without knowledge of the subject's exposure status) as negative, 1–9%, 10–49%, or ≥50%.

Mutation analysis

SSCP analysis of TP53 exons 5 through 9 was performed on all bladder tumor samples after a single pathologist (AS) microscopically examined each tumor and the diagnosis was confirmed. Each sample was individually rated for the percentage of the sample on the slide/block that was tumor; that is, the fraction of tumor to normal tissue was estimated for each sample. In this fashion, maximal tumor tissue was obtained for DNA extraction to ensure the highest sensitivity of the mutation analysis. Exons were amplified by PCR containing fluorescence dye labeled primers. Previously reported primer sequences for each exon were used.[25]

One microliter of PCR product and 1 μl of size standard TAMRA-350 (Applied Biosystems, Foster City, CA) were denatured in 4 μl of formamide/blue-dextran denaturing buffer at 95°C for 5 min and then loaded onto MDE gels. Gel electrophoresis was carried out on a DNA sequencer (ABI Prism 377, Applied Biosystems) with an external cooling system (Thermo NESLAB, Portsmouth, NH) attached for gels run at 25°C. Genescan 3.1 software (Applied Biosystems) was used for fragment analysis. Samples with variant SSCP bands were reamplified and purified using Centri-Sep columns (Princeton Separations, Adelphia, NJ). These were then directly sequenced in both directions by a DNA autosequencer (ABI Prism 377) using the Big Dye Terminator v3.0 sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The data were analysed with the Sequencing Analysis 3.3 software (Applied Biosystems) and Sequencher 4.1 software (Gene Codes, Ann Arbor, MI).

Statistical analysis

We evaluated the associations between exposure history (cigarette smoking, occupation, hair dye use and arsenic) and *TP53* status using logistic regression analysis, with adjustment for age, sex and extent of disease (*in situ,* noninvasive-low grade, noninvasive-high grade, invasive). For smoking history, we examined both recency of use (*e.g.,* never smoker, former smoker, current smoker) and pack-years smoked (duration smoked times average packs/day smoked). The cutoffs used for statistical analysis of arsenic were determined by analyzing the distribution among the age- and gender-matched companion control population studied.[21] A designation of "high" exposure was assigned to those in the 90th percentile (0.2 mcg/g toenail arsenic). For occupational history, we identified occupations related to risk of bladder cancer in our case-control analyses[26] and created a composite "at risk" variable for men and women separately. These included the following occupations in men: metal/plastic-processing machine operators (SOC code 754), fabricators, assemblers, hand workers (SOC code 77) and tractor-trailer truck drivers (SOC code 8212). In women, these were: retail sales (SOC code 43), kitchen workers/food preparation (SOC code 5217) and health service occupations (SOC code 523). We estimated relative risks for *TP53* mutation (yes, no) and *TP53* inactivation (<50% or ≥50% positively stained cells) separately as well as combined. Specifically, we assessed the relationship between exposure history and TP53 protein dysregulation in the absence of mutation, and *TP53* mutation with TP53 protein dysregulation. Lastly, we examined the distribution of type of mutations (transitions, transversions) according to exposure status. All *p*-values represent 2-sided statistical tests with statistical significance at $p < 0.05$.

Results

We obtained pathology materials on 416 (92%) of the 453 interviewed cases. An additional 6 cases had only cytologic material available and were not rereviewed. Of those rereviewed, 11 (3%) were deemed noncancerous by the study pathologist and 3 (<1%) could not be adequately evaluated. In addition to these cases, we excluded 1 subject who had multiple tumors of 2 histologies and 17 nonwhite subjects. Of the remaining 384 cases, 357 (93%) were investigated for *TP53* gene alteration (1 tumor was analyzed for mutation and not analyzed for IHC staining) and a total of 330 were successfully analyzed for exons 5–9. There were no significant clinical or demographic differences comparing those patients included and excluded for *TP53* study (data not shown). Further, based on the slide rereview, the percentage of tumorous tissue was 70.4% on average (median = 80%) and was unrelated to the measured presence of either persistent p53 protein measured by IHC ($p = 0.21$) or *TP53* mutation ($p = 0.70$). Roughly 80% (23/30) of tumors with *TP53* mutation displayed persistent staining.

Both *TP53* mutation prevalence and *TP53* inactivation (measured by persistent IHC staining) were less common among men and among older cases, but these differences were not statistically significant (Table I). Presence of *TP53* mutation and to a lesser extent protein staining increased with severity of disease (Table I). The presence of *TP53* alterations did not consistently differ according to smoking status or pack-years smoked (Table II).

TP53 mutation was more prevalent among men who held "at risk" occupations than those who did not hold these occupations, especially in the presence of *TP53* overexpression (Table II). Specifically, odds ratios (ORs), adjusted for age and stage, were elevated for male molding/casting machine operators (SOC Code 7542, a subset of 754) (OR for *p53* mutation = 2.5, 95% CI 0.5–13.0; OR for *p53* overexpression = 6.0, 95% CI 0.7–48.6), fabricators, assemblers, hand workers (SOC code 77) (OR for *TP53* mutation = 2.0, 95% CI 0.5–7.8; OR for *TP53* overexpression = 1.5, 95% CI 0.6–4.1) and tractor-trailer truck drivers (SOC code 8212) (OR for *p53* mutation = 1.9, 95% CI 0.6–6.2; OR for *TP53* overexpression = 1.3, 95% CI 0.6–2.9). In women, the age- and stage-adjusted ORs did not consistently differ according to history of an "at risk" occupation. This included retail sales (OR for *TP53* mutation = 0.6, 95% CI 0.1–3.0; OR for *p53* overexpression = 0.6, 95% CI 0.2–1.6 and health service occupations (OR for *TP53* mutation = 1.0, 95% CI 0.1–10.8; OR for *TP53* overexpression = 0.6, 95% CI 0.1–2.7). For kitchen workers/food preparation, it was only possible to estimate the OR for TP53 protein expression; the OR was slightly increased but with wide confidence intervals that included unity (OR = 1.6, 95% CI 0.3–9.2).

We observed a higher prevalence of *TP53* mutation and TP53 protein nuclear accumulation among subjects who reported hair dye use. The adjusted odds ratio for the association of hair dye use with *TP53* mutation and persistent staining was 4.1 (Table II; 95% CI 1.0–17.0). In men, the OR for the association of hair dye use

TABLE I – SELECTED PATIENT AND TUMOR CHARACTERISTICS FOR ALL CASES ACCORDING TO PRESENCE OR ABSENCE OF *TP53* MUTATION AND *TP53* INACTIVATION MEASURED BY IMMUNOHISTOCHEMISTRY

Variable of interest	*TP53* mutant		*TP53* inactivated	
	No. (%) (n = 300)	Yes (%) (n = 30)	No. (%) (n = 20)	Yes (%) (n = 236)
Sex				
Women	65 (21.7)	10 (33.3)	25 (20.8)	58 (24.6)
Men	235 (78.3)	20 (66.7)	95 (79.2)	178 (75.4)
Age, years				
≤55	65 (21.7)	8 (26.7)	23 (19.2)	55 (23.3)
>55	235 (78.3)	22 (73.3)	97 (80.8)	181 (76.7)
Stage				
Carcinoma *in situ*	9 (3.0)	1 (3.3)	2 (1.7)	8 (3.4)
Noninvasive, low grade	189 (63.0)	9 (30.0)	68 (56.7)	150 (63.6)
Noninvasive, high grade	25 (8.3)	6 (20.0)	17 (14.2)	15 (6.4)
Invasive	77 (25.7)	14 (46.7)	33 (27.5)	63 (26.7)
Histology				
Transitional cell carcinoma	296 (98.7)	29 (96.7)	118 (98.3)	233 (98.7)
Other	4 (1.3)	1 (3.3)	2 (1.7)	3 (1.3)

TABLE II – ODDS RATIOS (OR) AND 95% CONFIDENCE INTERVALS (CI)[1] FOR *TP53* MUTANT AND *TP53* INACTIVATED ASSOCIATED WITH EXPOSURE HISTORY

Exposure	Total (n = 357)[2]	*TP53* mutation (n = 330) OR (95% CI)	TP53 immunohistochemistry (IHC) inactivation (n = 356) OR (95% CI)	*TP53* mutation/IHC inactivation (n = 125)[3] OR (95% CI)
Smoking history				
Never smoker	57	1.0 (ref)	1.0 (ref)	1.0 (ref)
Former smoker	184	0.5 (0.2–1.4)	1.4 (0.7–2.6)	0.7 (0.2–2.9)
Current smoker	115	0.6 (0.2–1.8)	1.3 (0.7–2.6)	1.1 (0.3–4.4)
Pack-years of smoking				
Never smoker	57	1.0 (ref)	1.0 (ref)	1.0 (ref)
≤30	99	0.5 (0.2–1.7)	1.2 (0.6–2.5)	0.8 (0.2–3.7)
31–54	99	0.6 (0.2–1.9)	1.4 (0.7–2.9)	1.0 (0.2–4.4)
≥55	96	0.4 (0.1–1.4)	1.4 (0.7–2.8)	0.7 (0.1–3.4)
High-risk occupation,[4] men				
No	210	1.0 (ref)	1.0 (ref)	1.0 (ref)
Yes	64	2.9 (1.1–7.6)	1.7 (0.9–3.2)	4.1 (1.2–14.7)
High-risk occupation,[5] women				
No	36	1.0 (ref)	1.0 (ref)	1.0 (ref)
Yes	47	0.7 (0.1–3.1)	0.6 (0.2–1.8)	1.1 (0.1–9.4)
Hair dye use				
Never use	282	1.0 (ref)	1.0 (ref)	1.0 (ref)
Ever use	73	1.4 (0.4–4.4)	3.2 (1.4–7.2)	4.1 (1.0–17.0)
Toenail arsenic (μg/g)				
<0.2	310	1.0 (ref)	1.0 (ref)	1.0 (ref)
≥0.2	31	0.4 (0.1–2.9)	0.6 (0.3–1.4)	0.3 (0.1–2.3)

[1]Adjusted for age, sex, and stage (see text).–[2]357 subjects have *TP53* mutation data and/or TP53 IHC data.–[3]205 subjects who were *TP53* mutation negative with TP53 inactivation or p53 mutation positive without p53 inactivation are excluded from this analysis.–[4]High-risk occupations among men include metal/plastic processing machine operators (SOC code 754, n = 13); fabricators, assemblers, hand workers (SOC code 77, n = 22); and tractor-trailer truck drivers (SOC code 8212, n = 34). Some subjects are included in more than one SOC code.–[5]High-risk occupations among women include retail sales (SOC code 43, n = 36); kitchen workers/food preparation (SOC code 5217, n = 10); and health service occupations (SOC code 523, n = 10). Some subjects are included in more than one SOC code.

with *TP53* mutation alone was 0.7 (95% CI 0.1–6.2), whereas in women the estimated OR for mutation alone was 2.6 (95% CI 0.4–16.0). For immunohistochemical detection of *TP53* inactivation alone, the odds ratio for the association of *TP53*-positive staining and hair dye use in men was 1.7 (95% CI 0.5–5.4) and in women was 5.8 (95% CI 1.9–17.3).

Arsenic exposure, assessed using toenail measurements categorized into "low" and "high" exposure levels, was inversely related to the prevalence of *TP53* mutations; indeed, only 1 of the 31 patients with "high" arsenic biomarker levels had a *TP53* mutation (OR = 0.4, 95% CI 0.01–2.9; Table II). Also, a smaller proportion of tumors from individuals with "high" arsenic concentrations contained *TP53* inactivation (Table II) (OR = 0.6, 95% CI 0.3–1.4).

In our series, 6 of 33 (18%) of the observed mutations occurred in previously reported "hot spots" including codons 248, 280, 285 and 273,[8,17,27], and there were no mutations observed at codon 273 (Table III). Overall, the majority of the observed mutations were transversions (64%) (Table IV). There were no appreciable differences in the type of mutation (*i.e.,* transversion or transition) by smoking history or occupation. Among hair dye users, only 2 of the 10 mutations observed in hair dye users were transitions, with the association primarily for transversions (Table IV). As noted above, we found only 1 *TP53* mutation among the "high" arsenic exposure subgroup.

Discussion

To relate any particular pattern of exposure or risk factor to a certain pattern of somatic inactivation (*e.g.,* inactivation of the *TP53* pathway), it is essential to capture an unbiased sample of persons. Hence, our population-based approach is particularly well

suited to study the association of disease risk factors and *TP53* alteration and reflects a *TP53* mutation prevalence rate of other population-based series[13,28] and for earlier staged (*i.e.,* noninvasive) tumors.[28]

TP53 immunohistochemisty (IHC) is the most common approach used to determine the functional status of this gene in tumors. Most inactivating *TP53* mutations are missense and lead to an increased half-life of the protein, making mutant *TP53* detectable in tumors using IHC. Based on this fact, nuclear overexpression of *TP53* is commonly taken as a marker of *TP53* mutation despite the fact that some *TP53* mutations do not lead to overexpression (*i.e.,* nonsense) and that normal TP53 can also accumulate in cells in response to stress, and alterations in other members of the *TP53* pathway can alter protein processing and give rise to phenotypically identical persistent staining. In our work, there was a striking difference between the proportion of mutations found and the proportion of *p53* inactivation (Table I). The concordance rate (*i.e.,* percent agreement) between mutation and IHC nuclear accumulation has been reported to vary markedly, *i.e.,* from 7–90%,[29–38] but is generally between 65–75% across studies, including our own.[31] Interestingly, the pattern of some of these results tends to go in different directions depending upon whether one evaluates mutations or IHC. The precise reason for this is unclear but could relate to the specific part of the gene inactivated, as our data suggest that the concordance rate is specific to certain exons.

TABLE III – *TP53* MUTATIONS IN EXONS 5–9 IN BLADDER CANCER

ID	Exon	Codon	Base	Mutation	AA Change
1	5	151	451	C → T	Pro → Ser
2	5	158	472	C → T	Arg → Cys
3	5	173	517	G → C	Val → Leu
4	5	180	538	G → T	Glu → STP
5	5	180	538	G → T	Glu → Stop
6	5	183	548	C → G	Ser → STP
6	5	184	550	G → A	Asp → Asn
7	6	190	569	C → T	Pro → Leu
8	6	225	673	G → A	Val → Ile
9	7	235	703	A → T	Asn → Stop
10	7	236	673	A → G	Tyr → Cys
11	7	240	718	A → C	Ser → Arg
12	7	243	728	T → A	Met → Lys
13	7	248	742	C → A	Arg → Ser
14	7	248	743	G → A	Arg → Gln
15	7	248	743	G → A	Arg → Gln
16	7	248	744	G → C	Arg → Ser
17	7	250	748	C → A	Pro → Thr
18	7	253	757	A → C	Thr → Pro
19	7	255	763	A → T	Ile → Phe
19	7	259	775	G → A	Asp → Asn
20	8	276	827	C → T	Ala → Val
21	8	277	831	T → G	Cys → Trp
22	8	277	831	T → G	Cys → Trp
23	8	278	831	T → G	Cys → Trp
24	8	280	839	G → C	Arg → Thr
25	8	285	853	G → C	Glu → Asp
26	9	310	929	A → T	Asn → Ile
27	9	311	932	A → G	Asn → Ser
28	9	311	932	A → C	Asn → Thr
29	9	311	932	A → C	Asn → Thr
30	9	317	951	G → T	Gln → His
30	9	331	991	C → T	Gln → stop

Consistent with the largely hospital-based literature on bladder cancer,[8,15,17,39–43] we did not observe any significant association between tobacco smoking and *TP53* alteration. Studies with positive findings include a referral center-based study of 109 bladder cancers in which TP53 nuclear accumulation related to number of cigarettes smoked per day; however, no data were collected on *TP53* mutation.[39] In a single hospital-based series of 131 bladder cancer patients, Pacchioni *et al.*[40] reported an association of smoking with *TP53* expression but only in early stage disease. In our series, we did not detect a relative excess of *TP53* alterations associated with smoking in either noninvasive or invasive lesions (data not shown).

Previous reports of smoking "hot spot" mutations include codon 273,[17] codon 280,[8] and double mutations.[8,42] Consistent with our work, Schroeder *et al.*[43] reported no association of smoking with *TP53* mutation in 146 patients that constituted a subset of 245 patients recruited from 2 hospitals in North Carolina; however, current smoking was more strongly related to risk of tumors with G:C-A:T transitions at CpG sites than at non-CpG sites. Estimates by type of mutation in this study are somewhat imprecise because they are based upon a relatively small number of tumors. Also, the findings contradict those of lung cancer, for which G to T transversions are thought to be characteristic of tobacco exposure. The differences between the spectrum of *TP53* mutations in these tobacco-related solid tumors also may relate to multiple factors including the different classes of carcinogens (*i.e.,* PAHs *vs.* aromatic amines), the timing of *TP53* inactivation in clonal tumor development (*i.e.,* early *vs.* late, where there is misclassification of smoking-related mutations occurring after people who develop disease quit smoking) or differences in metabolism and delivery of tobacco carcinogens.

The prevalence of the specific types of *TP53* mutations vary among the tobacco-related tumors such as squamous cell carcinomas of the head and neck and esophageal cancer[44] and thus does not surprisingly differ for bladder cancers. One of the more recent and larger studies of bladder cancer found an association between the prevalence of *TP53* transition mutations and tumor stage,

TABLE IV – ODDS RATIOS (OR) AND 95% CONFIDENCE INTERVAL (CI)[1] BY TYPE OF MUTATION[2] AMONG BLADDER CANCER CASES

Exposure	No mutation (n = 300) No.	Transversions (n = 19)		Transitions (n = 12)	
		No.	OR (95% CI)	No.	OR (95% CI)
Smoking history[3]					
Never smoker	44	5	1.0 (ref)	3	1.0 (ref)
Ever smoker	256	14	0.4 (0.1–1.4)	9	0.6 (0.2–2.3
High-risk occupation[4]					
Males					
No	183	6	1.0 (ref)	5	1.0 (ref)
Yes	52	5	3.0 (0.9–10.8)	4	2.8 (0.7–10.9)
Females					
No	27	5	1.0 (ref)	1	1.0 (ref)
Yes	38	3	0.6 (0.1–3.1)	2	2.0 (0.1–32.5)
Hair dye use[3]					
Never use	242	11	1.0 (ref)	10	1.0 (ref)
Ever use	57	8	2.1 (0.5–8.7)	2	0.7 (0.1–4.9)

[1]*TP53* mutation negative is the "control" group.–[2]Two subjects with double mutations, one transversion and one transition appear in both columns. One mutation (G → deletion) was excluded.–[3]Age, gender and stage adjusted. One subject s missing information on hair dye use.–[4]Age and stage adjusted.

highlighting the problem of relying on series from small and unrepresentative populations. It is critical in evaluating the overall picture of *TP53* alterations to recall that even the IARC database, while extremely valuable, does not reflect the general population or permit evaluation of early stage lesions, which comprise the vast majority of bladder cancers worldwide. In our population-based study, we were able to control for tumor stage; however, the uniqueness of this design proves to limit comparability with earlier investigations.

We found that tumors from men employed in high-risk occupations were more likely to contain *TP53* alterations, but we did not observe this tendency in women. In the referral center-based study by Zhang *et al.*,[39] *TP53* nuclear accumulation was more prevalent among bladder tumors of those who worked in dye/ink-related occupations and in cooking-related occupations. We were unable to evaluate specific exposures such as dye/ink exposure, however, we found a 60% greater prevalence of *TP53* nuclear accumulation among women kitchen workers involved in food preparation, albeit with limited statistical precision. The hospital-based study from North Carolina found occupational exposure to lubricating oils, welding and soldering materials and soot, along with gasoline attendant and truck, bus or taxi driver occupations associated with *TP53* mutation, although none of these results were statistically significant.[43] Interestingly, our "at risk" occupations for men encompassed related occupations including tractor-trailer truck drivers, and although statistically significant overall, we had limited statistical power to evaluate individual occupations or specific mutation groups. Thus, defining phenotypic forms of bladder cancer on the basis of *TP53* status in future investigations may reveal etiologic subtypes of tumors associated with occupational exposures.

One of the more striking observations in the data is the significant association of *TP53* inactivation with hair dye use. The estimate of risk for *TP53* inactivation associated with hair dye use was found primarily in women. Hair dyes have been studied for their association with bladder cancer,[4,45–47] and the study of Gago-Dominguez *et al*.[4] has provided the strongest evidence that some hair dyes may pose a risk for bladder cancer. The current case series is part of a case-control study where analysis of the data raises the possibility of an increased risk for bladder cancer among certain subgroups of hair dye users.[19] Hair dyes have been noted to contain putative bladder carcinogens,[48,49] and the association of use with *TP53* alteration suggests that there may be a genotoxic mechanism responsible for the observed hair dye-bladder cancer risk association in some epidemiologic studies. Also, aromatic amines contained in hair dyes would be expected to produce transition mutations, consistent with what we observed.[50,51] If this is the case, however, it implies that the aromatic amines in hair dyes are more potent, more readily absorbed or at higher dose than those found in tobacco in the United States as we did not find similar results for *TP53* mutation associated with tobacco smoking. Because our findings derive from a small number of cases overall, it is important to confirm them in other and larger population-based series.

Finally, we observed a nonsignificant inverse association of toenail measures of arsenic exposure with *TP53* inactivation in bladder tumors. New Hampshire is documented to have areas of high levels of arsenic exposure, particularly in private wells used by a large proportion of residents.[27] There is also a well-known excess of bladder cancers in this region,[2] leading to speculation that arsenic may be a contributor to this excess; preliminary results from our study also support this possibility.[52] Previous studies have found that arsenic-related bladder cancers and skin lesions do not have *TP53* mutations and that arsenic does not directly inhibit *TP53*.[17,53,54] Indeed, there is also experimental evidence supporting our observation of an absence of *TP53* mutations in arsenic-exposed individuals.[55] Thus, our data are in agreement with the hypothesis that arsenic contributes to bladder cancer through a pathway that does not involve *TP53* mutation or inactivation that leads to persistent staining. Clearly, further data at low exposure levels are needed.

However, even in our large series, we should note that we had limited statistical precision in several analyses, particularly when evaluating *TP53* mutations. Our study population provided 80% power to detect a 2-fold difference between *TP53* IHC positive *vs*. negative cases for exposure prevalences of 25% or greater. For *TP53* mutation, we had 80% power to detect a 3-fold risk for exposure prevalences of 25% or more. Analyses of the subgroup of patients with both *TP53* mutation and inactivation or specific mutations were even more restricted.

In summary, our study represents an attempt to integrate *TP53* alteration in the disease classification of our population-based sample of bladder cancer patients. Our findings suggest that exposures to specific bladder carcinogens influence *TP53* mutations and *TP53* pathway inactivation and that characterizing these alterations will help clarify the etiology of bladder malignancies in the general population.

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